ANTIGEN

The present invention relates to a novel antigen from *Pseudomonas aeruginosa*, its use in medicine, particularly in the preparation of vaccines and in diagnosis.

P. aeruginosa is a Gram-negative aerobic motile bacterium with the form of rods. It is an environmentally ubiquitous, extracellular, opportunistic pathogen that causes significant morbidity and mortality in compromised subjects. Infection is of particular significance in subjects with cystic fibrosis, burns, chronic bronchitis, bronchiectasis and cancer.

Identification of immune responses, the search for vaccine candidates and suitable components for diagnostic tests have focused on components of *P. aeruginosa*. The outer membrane of *P. aeruginosa* contains toxins, including the lipopolysaccharide endotoxin, phospholipid and proteins. The various outer membrane proteins (Opr) of *P. aeruginosa* have been assigned an alphabetical naming system. While several proteins have been characterised by this scheme, the expression of some is only transient and highly dependent upon nutrient availability, culture conditions and the presence of antibiotics. Presently, three major Oprs, designated F,H2 and I, are recognised as antigenically common to and expressed in high copy numbers in all strains of *P. aeruginosa*.

We have now identified a protein from an outer membrane preparation of *P. aeruginosa*, which we have designated Pa60. The amino-terminal sequence of this protein does not demonstrate any sequence homology with other previously characterised proteins (GenBank data search). This protein is antigenic and is capable of inducing a protective immune response resulting in enhanced clearance of *P. aeruginosa*.

Thus, in a first aspect the present invention provides a protein antigen from *P. aeruginosa* and having a molecular weight in the range of about 60kDa to about 65kDa, as determined by SDS-PAGE.

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~~In a preferred~~ embodiment ~~the protein has the following~~ N-terminal sequence:

?-E-E-K-?-?-L-?-?- ?- ?- ?- ?- ?- V- V- ?- N- A (SEQ ID NO.1) and

preferably:

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~~?-E-E-K-T-P-L-T-T- A- A- ?- A- P- V- V- ?- N- A.~~ (SEQ ID NO:2)

Parts or fragments of the whole protein may themselves be antigenic and thus, in a second aspect, the present invention provides an antigenic fragment of the protein of the invention. In particular, the antigenic fragment will comprise the N-terminal sequence as described above.

The skilled man will appreciate that some variation in the sequence of fragments will be possible, while still retaining antigenic properties. Methods well known to the skilled man can be used to test fragments and/or variants thereof for antigenicity. Such variants also form part of the invention.

The antigenic protein, or fragments thereof, of the present invention can be provided alone, as a purified or isolated preparation, or as part of a mixture with other *P. aeruginosa* antigenic proteins.

In a third aspect, therefore, the invention provides an antigen composition comprising one or more proteins of the invention and/or one or more antigenic fragments thereof. Such a composition can be used for the detection and/or diagnosis of *P. aeruginosa*. In one embodiment the composition comprises one or more additional *P. aeruginosa* antigens.

In a fourth aspect, the present invention provides a method of detecting and/or diagnosing *P. aeruginosa* which comprises:

(a) bringing into contact an antigenic protein, or antigenic fragment thereof, or an antigen composition of the invention with a sample to be tested; and

(b) detecting the presence of antibodies to *P. aeruginosa*.

In particular, the proteins, antigenic fragment thereof or antigen composition of the invention can be used to detect IgG antibodies. Suitably, the sample to be tested will be a biological sample, e.g. a sample of blood or saliva.

In a fifth aspect, the invention provides the use of an antigenic protein, antigenic fragment thereof or antigenic composition of the present invention in detecting and/or diagnosing *P. aeruginosa*. Preferably, the detecting and/or diagnosing is carried out *in vitro*.

The antigenic protein, antigenic fragment thereof or antigen composition of the invention can be provided as part of a kit for use in *in vitro* detection and/or diagnosis of *P. aeruginosa*. Thus, in a sixth aspect, the present invention provides a kit for use in the detection and/or diagnosis of *P. aeruginosa* comprising an antigenic protein, antigenic fragment thereof or antigen composition of the invention.

In addition, the antigenic protein or antigenic fragment thereof of the invention can be used to induce an immune response against *P. aeruginosa*. Thus, in a further aspect, the present invention provides the use of an

antigen of the invention, a fragment thereof or an antigenic composition of the invention in medicine.

In yet a further aspect the present invention provides a composition capable of eliciting an immune response in a subject which comprises a protein or one or more antigenic fragments thereof of the invention. Suitably, the composition will be a vaccine composition, optionally comprising one or other suitable adjuvants. Such a vaccine composition may be either a prophylactic or therapeutic vaccine composition.

The vaccine compositions of the invention can include one or more adjuvants. Examples of adjuvants well known in the art include inorganic gels such as aluminium hydroxide or water-in-oil emulsions such as incomplete Freund's adjuvant. Other useful adjuvants will be well known to the skilled man.

In yet further aspects, the present invention provides:

(a) the use of a protein or one or more antigenic fragments thereof of the invention in the preparation of an immunogenic composition, preferably a vaccine;

(b) the use of such an immunogenic composition in inducing an immune response in a subject; and

(c) a method for the treatment or prophylaxis of *P. aeruginosa* infection in a subject, which comprises the step of administering to the subject an effective amount of a protein, at least one antigenic fragment or an antigen composition of the invention, preferably as a vaccine.

Preferred features of each aspect of the invention are as

for each other aspect *mutatis mutandis*.

The invention will now be described with reference to the following example which should not be construed as limiting the invention in any way.

The examples refer to the figure in which:

FIGURE 1: shows SDS-PAGE analysis of Pa60.

EXAMPLE 1: Protein Purification

Pseudomonas aeruginosa bacteria~~, strain 385 (Pa385), were~~ harvested from overnight culture of ~~100~~ agar plates by scraping the plates followed by washing twice by centrifugation at 10,000 x g for 10min at 4°C. A crude outer membrane preparation was obtained by extraction of the outer membrane component with buffered Zwittergent 3-14 ~~detergent and ethanol precipitation~~.

The outer membrane extract was lyophilised and resuspended in starting buffer (20mM Tris, pH8.5). This preparation was subjected to anion exchange chromatography using a Q2 column (BioRad) and a sodium chloride gradient to elute the proteins. The fractions eluted from the column were initially assessed for protein content by analytical SDS-PAGE. From this was determined the elution of profile for Pa60 allowing fractions containing Pa60 to be collected from susequent runs for further purification. These fractions were dialysed against distilled water, lyophilised, resuspended in a minimal amount of distilled water and further dissolved in 4 times the volume of sodium dodecyl sulphate (SDS) reducing buffer (62.5mM Tris, pH6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) ß-mercaptoethanol, 1.2×10^{-3}% (w/v) bromophenol blue). The SDS preparation

was incubated at 37°C for at least 30min prior to being loaded onto the stacking gel of the electrophoresis column.

~~Pa60 was purified using preparative polyacrylamide~~ electrophoresis (PAGE). Preparative SDS-PAGE was performed using the BioRad model 491 Prep Cell using a 9% T-1.42% C acrylamide/BIS (N,N'-methylene-bisacrylamide) separating gel with a 10ml 4% T-0.36% C acrylamide/BIS stacking gel polymerised in a 28mm (internal diameter) column. Fractions eluted from the column were concentrated by lyophilisation and analysed for protein content by analytical SDS-PAGE. Pa60 isolated using these conditions contained SDS which was subsequently removed by potassium phosphate precipitation. fractions containing Pa60 were pooled and dialysed prior to ~~determination of protein concentration~~.

Analytical identification of the protein was performed by analytical SDS-PAGE using either gradient 10-15% or homogenous 12.5% acrylamide gels and coomassie or silver stained. Protein concentration was determined using the Pierce micro BCA assay.

Results

Pa60 was successfully separated from other *P. aeruginosa* proteins by the described method. Figure 1 shows the position of this protein on SDS-PAGE.

EXAMPLE 2: N-terminal sequencing of Pa60

Pa60 was prepared for N-terminal amino acid analysis by excising the region containing the protein from an SDS-PAGE gel. The gel segments were sent to both the Biomolecular resouirce facility, Australian National University, Canberra, Australia and MUCAB Services, Macquarie University, North ryde, NSW, Australia.

Results

An N-terminal amino acid sequence was obtained which identified sixteen of the first nineteen amino acids. Possible amino acids were identified for the remaining residues and where there was uncertainty with a probable identification.

SEQUENCE:

	1	2	3	4	5	6	7	8	9	10
Definite		E	E	K			L			
Probable					T	P		T	T	A
Possible	S				A	L/S		A	I/D	W

	11	12	13	14	15	16	17	18	19
Definite					V	V		N	A
Probable	A		A	P					
Possible	F/L	G/S	N	D					

~~This provides a sequence with the following definite~~ amino acids:

1-2-3-4-5-6-7-8-9-10-11-12-13-14-15-16-17-18-19

~~?-E-E-K-?-?-L-?-?- ?- ?- ?- ?- ?- V- V- ?- N- A~~ (SEQ ID NO. 1)

~~If one includes probable amino~~ acids the following sequence is obtained:

1-2-3-4-5-6-7-8-9-10-11-12-13-14-15-16-17-18-19

~~?-E-E-K-T-P-L-T-T- A- A- ?- A- P- V- V- ?- N- A~~ (SEQ ID NO. 2)

EXAMPLE 3: Bacterial clearance following immunisation in a rat model

Specific pathogen free male rats received an intra-Peyer's patch (IPP) immunisation on day 1 and the live bacterial challenge on day 14. The animals were sedated by anaesthesia. The small intestine was exposed through a mid-line abdominal incision and the antigen injected subserosal to each Peyer's patch using a 27-gauge needle. The immunisation protein (Pa60) was prepared by emulsification of 200 or 800μg of protein per ml in a 1:1 ratio of Incompatible Freund's adjuvant (IFA) and phosphate

buffered saline (PBS) and a total inoculum of 10 or 40μg of protein respectively was administered to each animal. Animals were challenged for 4 hours with live bacteria (bacteria count 5×10^8 CFU) 14 days after the immunisation. Bacteria were grown overnight at 37°C in 5% CO_2 on nutrient agar plates, recovered, washed and resuspended in PBS to the required concentration. Bacteria were introduced into the lungs via an intra-tracheal cannula and 4 hours later the rats were euthanased. Blood was collected and aliquots of serum stored at -20°C for antibody analysis. Lungs were lavaged by flushing with 5 x 2ml of PBS and the pooled lavage (BAL) assessed for bacteria numbers. Following lung lavage, the lungs were removed, homogenised and assessed for numbers of bacteria. Cytospin slides were prepared for determination of differential cell counts in the lung lavage. total cell numbers present in the lung lavage were calculated by staining with trypan blue and counting using a haemocytometer.

Results

Rats immunised with Pa60 and challenged with live bacteria of the Pa385 homologous strain on day 14 showed an enhancement of bacterial clearance. Rats immunised with both 10μg or 40μg Pa60 had fewer bacteria recovered in both the BAL and lung than the non-immune group after 4 hours (Table 1).

Greater numbers of phagocytic cells were present in the BAL of Pa60-immunised animals and correlated with the enhanced bacterial clearance in these animals (table 2).

Table 1: Pulmonary clearance following Pa60 immunisation and challenge with *P. aeruginosa* (strain 385)

RAT GROUP	n[b]	*P. aeruginosa* recovered 4h post-challenge (log_{10}CFU)[a]	
		BAL	LUNG HOMOG.
NON-IMMUNE	5	7.63±0.11	8.66±0.18
10μg Pa60	6	6.95±0.07	8.43±0.09
40μg Pa60	4	7.19±0.07	8.37±0.19

Table 2: Cell count of Phagocytes in BAL following bacterial challenge

ANIMAL GROUP	TOTAL PHAGOCYTIC CELLS IN BAL
NON-IMMUNE	1.2 (±0.3) x 10^6
10μg Pa60	4.3 (±1.2) x 10^6
40μg Pa60	7.4 (±1.7) x 10^6

EXAMPLE 4: clinical diagnostic study

Children from the Royal Children's Hospital in Melbourne that had been diagnosed with cystic fibrosis provided samples for this study. Bronchoalveolar lavage (BAL) abnd serum were collected over a 3-4 year period from patients from the time of diagnosis as an infant. The samples were divided into groups based on clinical status of *P. aeruginosa*.

Group 1: Non-cystic fibrosis controls (age matched children with Stridor);

Group 2: Negative for *P. aeruginosa*;

Group 3: Upper respiratory tract isolation of *P. aeruginosa*, negative *P. aeruginosa* in lower respiratory tract;

Group 4: Cleared *P. aeruginosa* in the lower respiratory tract (negative in the next BAL sample); and

Group 5: Positive for *P. aeruginosa* in consecutive BAL samples.

~~An enzyme linked~~ immunosorbent assay (ELISA) was used to measure antibodies to Pa60 in BAL and serum samples. Polysorb microtitre wells were coated with purified Pa60 at a concentration of 1μg per ml. The plates were washed five times in PBS containing 0.05% tween 20 between incubation steps. The wells were blocked with skim milk in PBS-0.05% Tween 20 for 60 min. Wells were incubated for 90 min with serum or BAL samples that were diluted in blocking buffer for analysis. Conjugated immunoglobulins used were rabbit anti-human IgG, IgA and IgM and wells were incubated with conjugated immunoglobulins for 90 min. The pltes were then developed. Human IgG, IgA and IgM were used to quantitate the antibody.

Results

An increase in antibody titre was observed as the incidence of infection with *P. aeruginosa* occurred. The non-cystic fibrosis control group and the non-infected cystic fibrosis patients had negligible titresto Pa60. Increased titres of IgG to Pa60 were observed, particularly in the patients with consecutive *P. aeruginosa* culture from the BAL (Group 5). In the BAL a significant increase in IgA titre was observed.

Table 3: Pa60-specific Antibody in Serum and Bronchoalveolar lavage from cystic fibrosis and non-cystic fibrosis children

PATIENTS	SERUM[a]			BAL[a]		
	IgG	IgA	IgM	IgG	IgA	IgM
GROUP 1	1.74	0.11	0.67	0.03	0.05	0.02
GROUP 2	1.40	2.34	2.10	0.03	0	0.02
GROUP 3	7.08 ±8.4	10.9 ±18	2.03 ±2.5	0.03 ±0.01	0.21 ±0.13	0.03 ±0.01
GROUP 4	18.9 ±21.9	0.56 ±0.6	1.46 ±2.07	0.02 ±0.01	0.12 ±0.04	0.01 ±0.01
GROUP 5	54.5 ±76	7.5 ±12.5	6.2 ±0.5	0.03 ±0.01	0.81 ±0.30	0.03 ±0.01

EXAMPLE 5:

Pulmonary Challenge of rats with *Pseudomonas aeruginosa* following mucosal immunisation with Pa60.

DA rats were immunised with Pa60 such that they received 10μg Pa60 administered to intestinal Peyer's Patches (IPP). The Pa60 was delivered emulsified in Incomplete Freund's adjuvant. Fourteen days post-IPP, all the immunised rats received an intra tracheal (IT) boost with 10μg Pa60 in phosphate buffered saline. Seven days post-IT boost, the immunised group and an untreated control group were challenged via IT administered of 5 x 10^8 CFU live *P. aeruginosa*. The rats were killed and samples collected for analysis at 4h post-challenge.

Results

Bacterial Clearance

Bacterial recovery in bronchoalveolar lavage (BAL) and lung tissue

		Bacterial Recovery ($\log_{10}$ CFU)*	
Group	n+	BAL	Lung
Non-immune	5	7.92 ± 0.11	9.01 ± 0.15
10μg Pa60	5	6.34 ± 0.08	7.58 ± 0.08

* Data expressed as mean ± S.E.M.

\+ number of animals

Rats immunised IPP with an IT boost at day 14 significantly cleared the *P. aeruginosa*

from both the BAL and lung tissue.

White Cell Recruitment to Infection

White cell count in BAL

Group	n	White cell count* $\times 10^6$
non-immune	5	6.8 ± 1.4
10μg Pa60	5	36.0 ± 5.0

* data represents mean ± S.E.M.

Rats immunised with Pa60 more rapidly recruited white cells to the lungs following bacterial challenge. Nearly all the white cells recovered in the BAL were either polymorphonuclear neutrophils (PMNs) or macrophages. The early recruitment of white cells to the bronchoalveolar spaces correlated with bacterial clearance.

Antibody Responses

Antibody in serum and BAL following mucosal immunisation with Pa60

Antibody in Serum

Group	n	IgG*	IgA*	IgM*
non-immune	5	0	0	14.8 ± 0.56
10μg Pa60	5	1310 ± 119	10 ± 2	100 ± 59

* IgG, IgA and IgM expressed as Elisa units

Serum antibody to Pa60 was detected in immunised rats. There were significant titers of IgG, IgA and IgM.

Antibody in BAL

Group	n	IgG*	IgA*	IgM*
non-immune	5	1.5 ± 0.2	0	0.5 ± 0.02
10μg Pa60	5	21.3 ± 2.6	7.8 ± 4.2	0.8 ± 0.3

* IgG, IgA and IgM expressed as Elisa units

Antibody to Pa60 was detected in the BAL in immunised animals. There were significant titers of both IgG and IgA specific for Pa60.

In a particular embodiment, this invention provides a kit for detection or diagnosis of *P. aeruginosa* in a sample from a patient. The kit contains at least one or more antigens or antigenic fragments according to this invention, along with the means to detect binding between the antigens or fragments and antibodies which specifically bind such antigens or fragments. Selection of suitable means for detecting antigen-antibody binding is easily within the skill of the ordinary worker in this art, and include primary and/or secondary labeled antibodies to IgG from humans or other mammals, and/or other known materials for sandwich assays, ELISA assays, competitive immunoassays, and other well known immunometric assay formats.

~~In yet another particular embodiment, this invention provides a method for diagnosing *P. aeruginosa* in a subject suffering from cystic fibrosis. This method comprises bringing into contact one of the proteins, antigenic fragments or antigen compositions disclosed in this invention with a biological sample obtained from a subject with cystic fibrosis. The biological sample is preferably a sample of mucous, e.g. saliva. This method further comprises detecting the presence of antibodies to P. aeruginosa in such a sample by, for example, detecting binding between the antigens or fragments and antibodies which specifically bind such antigens or fragments, using detection means which are of common knowledge to those of skill in the art.~~

add a^2